ABSTRACT

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CHARACTERIZATION OF ZIC2 AS AN ONCOPROTEIN IN PROSTATE CANCER

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The field of prostate cancer research is in need of biological markers that predict which cancers do not need treatment, those that can be treated successfully with a localized treatment and more specific cases in which patients are likely to have an aggressive form of cancer that will require more aggressive surgical and chemotherapeutic treatments. ZIC2 is one of five members of a family of proteins that play critical roles in neural crest and mesoderm growth in normal embryonic brain development and in the adult cerebellum of vertebrates. Found throughout the animal kingdom, ZIC1-5 genes encode five distinct ZIC proteins containing five highly conserved C2H2-type zinc finger motifs, whose structural integrity is important in carrying out its function as a transcription factor. We hypothesize that ZIC2 has functional significance at the molecular and cellular levels in the initiation of prostate adenocarcinoma (PRAD) and the progression to metastatic and/or castration resistant prostate cancer (CRPC). Bioinformatic predictions suggest that the function of ZIC2 is

regulated by post-translational modifications, such as phosphorylation, ubiquitination and sumoylation. This proposal further outlines the research hypothesis for investigating the role of ZIC2 in prostate cancer progression and the effects of the post-translational modification, ubiquitination, on the loss or gain of function of ZIC2.

CHARACTERIZATION OF ZIC2 AS AN ONCOPROTEIN IN PROSTATE CANCER

A DISSERTATION

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CHAPTER 1

INTRODUCTION

The National Cancer Institute's Surveillance, Epidemiology and End Results Program estimates that 180,890 new cases of Prostate cancer and 26,120 deaths in 2016.[1] This equates to a lifetime risk of 15%, i.e., 15 out of 100 men will be diagnosed with prostate cancer at some point during their lifetime. However, at least 80% of diagnoses have a low-risk of progressing to a life- threatening disease and do not require radical chemotherapeutic or surgical intervention. However, due to a lack of prognostic markers for disease progression, the majority of these low-risk patients undergo treatments leading to significant sexual side effects. The Prostate specific antigen (PSA) test is the current and popular screening tool used in medical diagnoses of prostate cancer.[2] However, PSA levels may not always be an accurate indicator of prostate cancer development.[3]

Current treatment methods for prostate cancer include prostatectomy, hormone therapy, radiation therapy, and chemotherapy with the use of sensitive drugs.[4] The focal point of prostate cancer hormonal treatment is Androgen deprivation therapy (ADT).[5] Prostate development utilizes hormones for cellular regulatory needs.[6]

Male androgens- testosterone and dihydrotestosterone (DHT) are the main ligands for androgen receptor signaling stimulation, the Androgen receptor pathway is a major target for prostate cancer treatment due to the ease of depriving the receptor of the ligand that is positively transducing a signal once activated. This process allows for the male hormones which stimulate healthy and prostate tumor growth to be depleted and then restored once the cancer has been annulled.[5] ADT as a treatment option and in some cases when paired with radiation therapy has had favorable outcomes for patient survival but the core issue is the relapse during or after ADT has been discontinue.[7] This phase of relapse is considered as Castration Resistant Prostate Cancer (CRPC).[8] Gene expression microarray has identified a number of genes that are significantly up regulated in increasing abundance during the multi-step progression model initiating in prostatic intraepithelial neoplasia and progressing through CRPC. We reason that these genes are likely to play clinically significant roles in predicting disease progression.

Among those genes that are up regulated in prostate cancer, is the gene encoding the C2H2- zinc finger containing transcription factor, *Zic family member 2 (ZIC2).* Furthermore, an inverse correlation was observed between increased ZIC2 expression and decreased expression of Androgen Receptor signaling gene targets in the same data set (Figure 1). Additional RNAseq information has provided similar results for ZIC2 in PCa. The Cancer Genome Atlas data shows a comparison of normal to cancer with ZIC2 being highly upregulated in prostate adenocarcinoma.

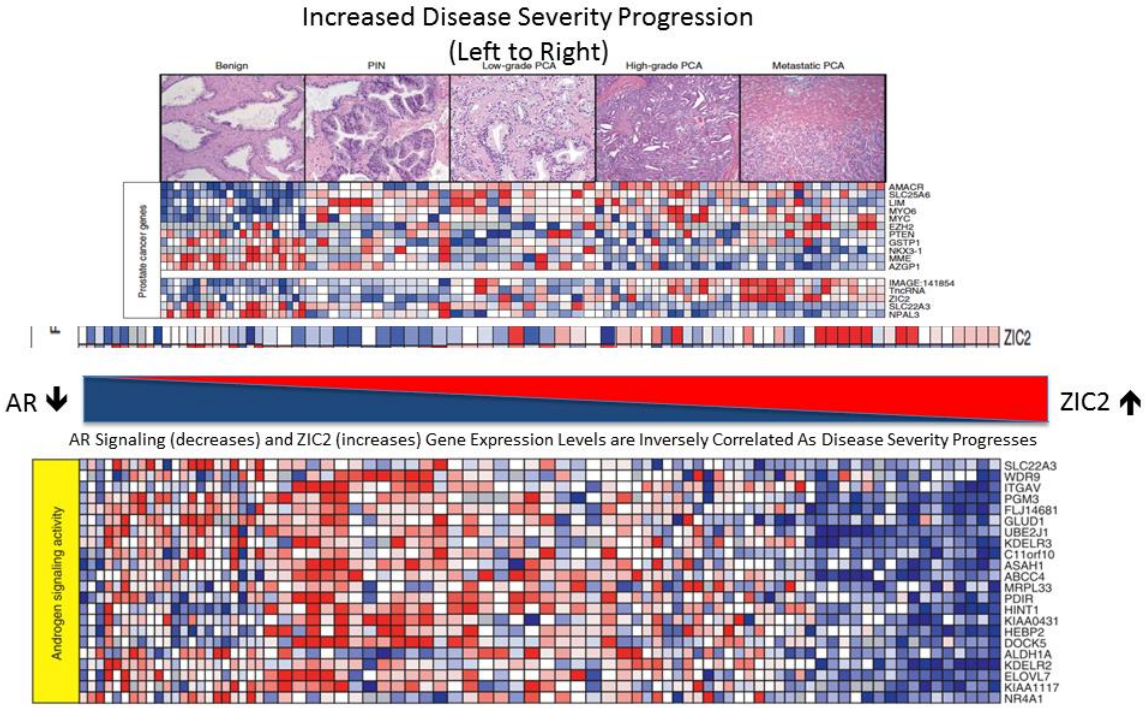


Figure 1. ZIC2 and Androgen receptor are observed to have contrasting expression in bioinformatics analysis.

Source: Adapted from Tomlins, S. A.; Mehra, R.; Rhodes, D. R.; Cao, X.; Wang, L.; Dhanasekaran, S. M.; Kalyana-Sundaram, S.; Wei, J. T.; Rubin, M. A.; Pienta, K. J., Integrative molecular concept modeling of prostate cancer progression. *Nature genetics* **2006,** *39* (1), 41-51.

Tomlins et al. utilized a bioinformatic approach to classify genes associated with prostate cancer.[9] The outcome of the integrative analysis was also profiled against increasing histological grades of prostate cancer. The tumor grade analysis expanded from benign prostatic hyperplasia to metastatic prostate cancer. There was a significant correlation exhibited through little to no expression of ZIC2 and increasing with progression of worsened disease status. Additional RNAseq information has provided similar results for ZIC2 in prostate cancer. TCGA data shows a comparison of normal to cancer with ZIC2 being highly upregulated in prostate adenocarcinoma. The overall hypothesis is the aberrant overexpression of ZIC2 contributes to the progression of

Prostate Cancer. Thus, reduced expression of ZIC2 will negatively affect cell proliferation and disrupt other hallmarks of cancer. The following objectives have been identified to test the hypothesis:

Objective 1: To characterize the functionality of ZIC2 with the implementation of CRISPR-*Cas9*; our lab has characterized steady-state expression among varying prostate cancer cell line models. Moving forward, it is our goal to characterize the functionality of ZIC2 following a forced knockout using CRISPR-*Cas9* vectors.

Objective 2: To assess the metabolic activity after *ZIC2* CRISPR-*Cas9* gene editing in PC-3 cells; cellular metabolism is a signature in cancer models and a preference between cellular respiration and fermentation is a distinct indication of cancer suppression or promotion.

Objective 3: To determine the stabilization activity of post-translational modifications on ZIC2; to date, ZIC2 has been reported to be phosphorylated. It is our goal to alleviate the effects of ZIC2 on prostate cancer progression, discovered in Objective 1, by disrupting the predicted post-translational modification sites.

CHAPTER 2

LITERATURE REVIEW

ZIC family member 2 is one of five members that make up the family of ZIC proteins (Figure 2).[10-11] The entire ZIC family comprises transcription factors that have been reported to play various roles in neuronal and embryonic development.[10, 12-13] Each ZIC family member has five zinc fingers that with the C2H2 motif.[14] The zinc finger domains allows for DNA binding and protein-protein interactions.[15] ZIC family members 1-3 have a ZIC/ odd-paired conserved (ZOC) region, which play an essential role in protein-protein interactions.[13]

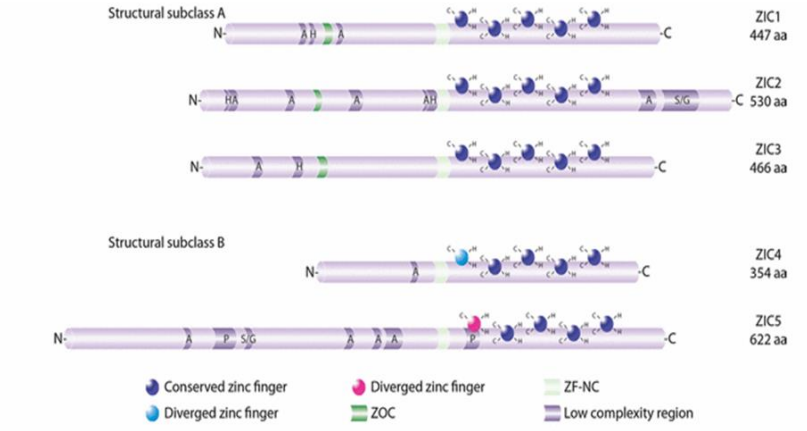


Figure 2. Structural differences of the Zic family members when comparing ZIC2.

Source: Adapted from Houtmeyers, R.; Souopgui, J.; Tejpar, S.; Arkell, R., The ZIC gene family encodes multi-functional proteins essential for patterning and morphogenesis. *Cellular and molecular life sciences: CMLS* **2013**, 70 (20), 3791-811.

Mizugishi et al. elucidated a protein-protein interaction between ZIC2 and other regulatory transcription factors, such as GLI family zinc finger proteins.[11] The predicted molecular weight of ZIC2 is 55kDa. Our results indicate a difference in molecular weight (65kDa), which suggests the occurrence of post-translational modifications.

The ZOC motif was identified as a regulatory domain and the responsible binding location interacting with I-mfa (now referred to as MyoD family inhibitor, MDFI).[16] Binding of MDFI to ZIC2 occurred at the N-terminus of ZIC2 and caused inhibition of ZIC2 transcriptional activity. An additional common structural characteristic amongst the family members is the Zinc finger N-terminally conserved domain (ZF-NC).[13] This domain is immediately upstream of the zinc fingers and its approximate length is 14-21 amino acids.[13]

ZIC2 has been implicated in a wide variety of embryonic development processes: neurulation, gastrulation, body pattern formation, axon guidance, and forebrain development.[13-14, 17-19] During brain development, a mutation in *ZIC2* promotes, Holoprosencephay, abnormal forebrain development.[20] Holoprosencephaly (HPE) is a disease in which the brain forms into a unilateral organ instead of the normal left and right hemispheres.[21-23]

ZIC2 occupies a specific region on the 13q chromosome. Deletions and duplications of chromosome 13 have been reported to contribute to HPE. Brown et al. reported 16 mutations with in a group of HPE patients.[21] Within this study, mutations #10-#16 emphasize a common alanine tract expansion. An additional group of 7 exclusive patient mutations resulted in frameshifts contributing to loss-of function. ZIC2

also contains two histidine tracts near the N-terminus. The most impactful histidine tract possesses 9 residues. Dubourg et al. identified ZIC2 mutations leading to HPE in 200 patients.[24] Seven mutations within the cohort were identified specifically related to ZIC2. Within the seven, two of the mutations consisted of poly-histidine tract polymorphisms. One of the tracts lacked a histidine leading to eight histidine residues while the other tract contained an additional histidine, leading to ten residues. The histidine tract polymorphisms within ZIC2 have been linked to neural tube defects.

ZIC proteins are hypothesized to regulate transcriptional activity via direct DNA binding.[25] Sakurada et al. investigated the proteins from rat brain nuclear extracts responsible for activation of the Ca^{2+}/calmodulin-dependent protein kinase II (α-Cam kinase II) promoter.[26] Construction of a cDNA library of brain extracts identified ZIC2 as a binding protein. The activity of the α-Cam kinase II promoter was up-regulated up to 5 fold in cultured neuronal cells by ZIC2.[26]

Aside from direct DNA binding, ZIC family members can function as co-factors through the formation of complexes with other transcription factors and proteins.[15, 27-28] Aruga et al. investigated the transcriptional activation of two complexes that were ZIC2 dependent.[15] Complex I was a compilation of a DNA dependent protein kinase catalytic subunit (DNA-KPCs), Ku70/80, and poly (ADP-ribose) polymerase; DNA KPCs and Ku70/80 are collectively responsible for double strand break repair. The Complex II consisted of Ku70/80 and RNA helicase. Results showed that both complexes depend on ZIC2 for DNA binding upstream of the target gene promoter. Salero et al. reported on (ZIC1 and) ZIC2 binding to the gene promoter of Apolipoprotein E (ApoE).[29] A cDNA

library was constructed of human brain samples. The library data was a partnership between other assays, which included yeast one hybrid system, Electromobility shift and luciferase reporter activity to confirm the transcriptional activation of three binding sites for ZIC family members 1 and 2. The binding sites within the Apolipoprotein E (ApoE) promoter were concluded to be a consensus nucleotide sequence-CTG followed by a GC-rich region. ApoE is an important protein in transport and metabolism of plasma cholesterol and triglycerides.

Pan et al. identified ZIC2 and other family members to have influential roles in regulating Myogenic factor 5 (Myf5).[27] During embryonic development, Myf5 and other myogenesis proteins give support to muscular tissue formation. A myogenic protein can be inhibited specifically by inhibitor of MyoD family (I-mfa) protein. Mizugishi et al. demonstrated the ability of I-mfa to act as a transcriptional repressor to ZIC2.[16] The repressor activity of I-mfa disrupts nuclear translocation of ZIC2 and restricts it to the cytoplasm. This repression is achieved via N-terminus binding of ZIC2 to the C-terminus of I-mfa. I-mfa inhibits nuclear localization of ZIC 1, 2 and 3.

Cell fate determination, motility, and organogenesis can be regulated by way of canonical and non-canonical Wnt signaling.[30] Pourebrahim et al. reported ZIC2 as a regulator of the β-catenin-TCF4 complex.[28] In canonical Wnt signaling β-catenin is activated and retained in the cytoplasm. The TCF proteins aid in the translocation of β-catenin to the nucleus. ZIC2 was shown to bind to TCF4 via the DNA binding high mobility group box. This resulted in restriction of the β-catenin nuclear translocation.

mRNA expression of ZIC 1, 2, and 5 have been identified in meningiomas and other brain tumors, such as medullablastoma and glioblastoma.[31] Previous research of rat brain development has categorized ZIC family members to be required for cell proliferation and differentiation of meningeal progenitor cells. Meningiomas are primary tumors of the central nervous system. RT-PCR and microarray data confirmed higher expression of ZIC2 meningiomas. Thus, concluding that ZIC2 is a potential novel molecular marker for meningiomas.

Marchini et al. investigated a role for ZIC2 in epithelial ovarian cancer.[32] ZIC2 mRNA was expressed more in the malignant tumors of epithelial ovarian cancer compared to those with low malignant potential. It was concluded that ZIC2 may be a distinguishable candidate for helping to identify tumor outcomes in epithelial ovarian cancer. In an effort to further classify ZIC2 as an oncoprotein, we hypothesize a role for ZIC2 in migration and invasion of tumor metastasis modeled by in vitro experimentation. ZIC2 knockdown impaired the dispersion and migration of Cajal-Retzius cells into the cortical layers of embryo forebrain.[33] Chan et al. explored the relationship between ZIC2 and GLI1 in cervical cancer cells.[34] Overexpression of ZIC2 in cervical cancer cells increased cell proliferation, hedgehog signaling activity and anchorage-independent growth ability. Additionally, siRNA mediated ZIC2 knockdown induced cell apoptosis while ZIC2 overexpression enhanced proliferation in pancreatic cancer cells.[35] In totality, these studies contribute to growing knowledge of a role for ZIC2 in proliferation, migration and invasion.

In this age of personalized medicine where unique genetic alterations are touted as potential therapeutic targets, there is still the common observation, first reported by Otto Warburg, that most cancer cells produce energy by high rates of glycolysis followed by lactic acid production, instead of lower rates of glycolysis followed by oxidation of pyruvate in mitochondria as in most differentiated cells.[36-37] Aerobic glycolysis yields less ATP per glucose molecule. However, it is the preferred energy metabolism used by cancer cells that need to generate many intermediate metabolites from glucose for cell growth and do not want to oxidize glucose completely to CO_2. Warburg hypothesized that mitochondrial dysfunction itself was the cause of cancer.[38] Currently this has been replaced with the idea that mutations to oncogenes and tumor-suppressors are the cause of cancer and that Warburg's observations were merely an effect. However, many experiments continue to support the notion that cancer can be treated by increasing mitochondrial oxidative phosphorylation.

Mitochondria are the energy control center of the cell.[39] The ATP production for continued cellular activity is generated by glycolysis and oxidative phosphorylation. The electron transport chain resides within the inner membrane of the mitochondria and oxygen begins the proton exchange throughout complexes I, II, III, IV, and V (Figure 3). Glycolysis, the process of breaking down glucose, yields pyruvate for entry into the citric acid cycle.[8] Pyruvate oxidation generates the bulk of ATP molecules necessary for cellular energy.

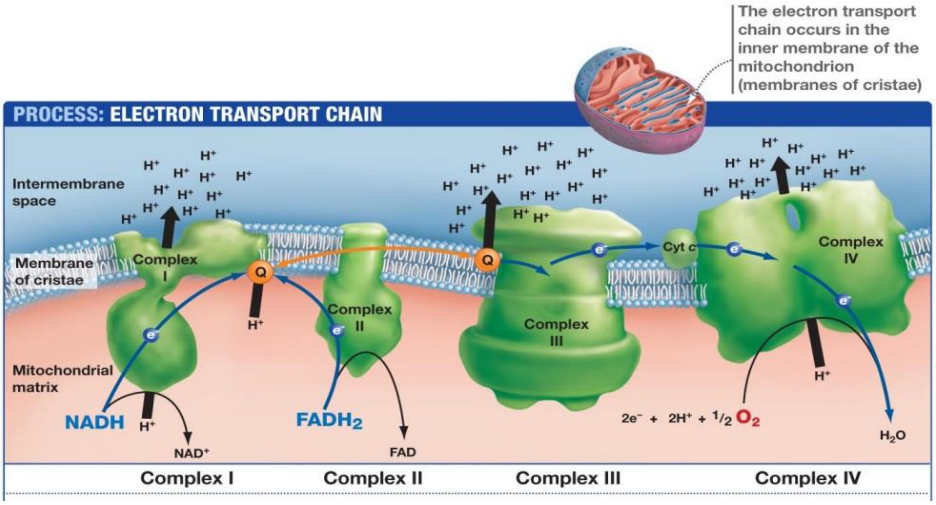


Figure 3. The process of electron transfer in the electron transport chain.

Source: Adapted from Biochemist01 Archives Page. https://biochemist01.wordpress.com/tag/electron-transport-chain/ (accessed Oct 4, 2015).

The Warburg effect is an energy generating process that supports glycolysis to feed into lactic acid fermentation in the cytosol. Aerobic glycolysis yields less ATP molecules for energy usage however it is the preferred energy metabolism way of life for cancer cells. The mitochondrial dysfunction within cancer cells is an escape mechanism to avoid apoptotic triggers.[40-41] Mitochondrial dysfunction has been reported in several disease models including cancer and neurodegenerative disorders.[40, 42-47] In an effort to perpetuate invasiveness and continued cell divisions, the metabolic transformation is useful for generating energy as a means of maintenance. The Warburg effect is the metabolism process cancer cells utilize to survive in low-oxygen, hypoxic, conditions, shown in Figure 4. This process is preferred over the alternative-oxidative phosphorylation. Cancer cells undergo a metabolic transformation that contributes to the

hypoxic environment by producing excess amounts of reactive oxygen species (ROS).[48]

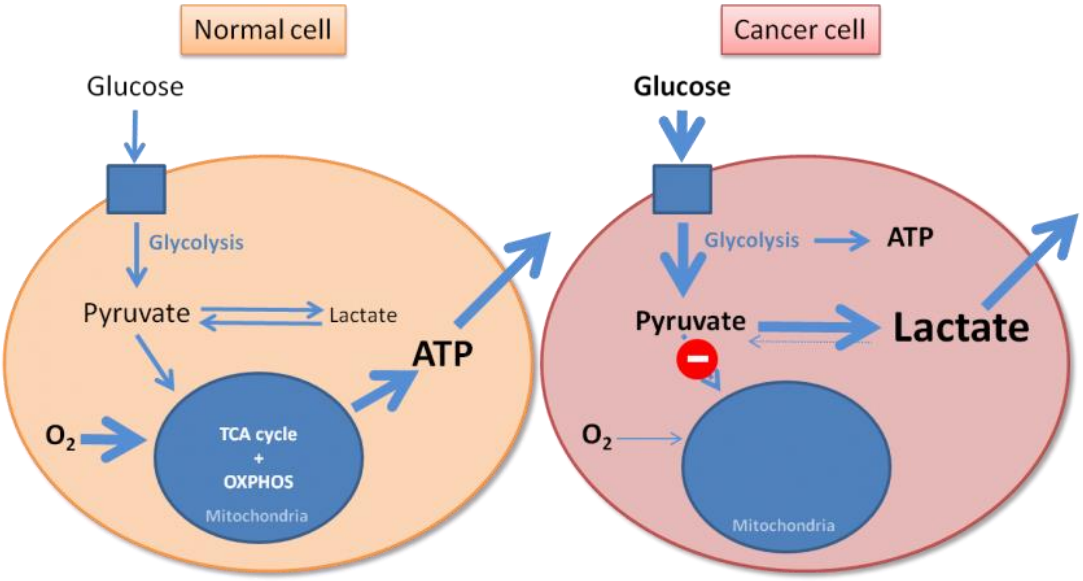


Figure 4. Comparison of ATP production in normal and cancer cells.

Source: Adapted from Metabiolab. http://metabiolab.com/about-6/technologies-suite/ (accessed Sept 20, 2015).

Kloss-Branstatter et al. evaluated somatic mutations associated with several genes encoded by the mitochondrial DNA contributing to prostate cancer tumor progression and elevated PSA levels.[49] Ling et al. performed a compelling study in human H460 non-small cell lung cancer cells to identify the role of mitochondria in Bortezomib induced apoptosis.[50] H460 cells were treated with Bortezomib, an anti-tumor drug and inhibitor of the 26S proteasome activity. Within 24 hours of treatment, increased ROS production, increased mitochondrial membrane potential and release of cytochrome c were observed supporting a role for Bortezomib in restoring mitochondrial mediated apoptosis. Kaipparettu et al. used cytoplasmic hybridization to study the effects of mitochondria derived from benign and malignant breast tumor cells on the oncogenic properties of

osteosarcoma cells.[47] The introduction of mitochondria from benign breast cells into osteosarcoma cells reinstated respiratory chain activities and elevated ATP synthesis, whereas mitochondria from breast cancer cells did not. The same lab has shown that while the presence of mitochondria in the osteosarcoma cells is essential for tumor formation in mice, the introduction of mitochondria with mild mutations conferring varying levels of reduced oxidative phosphorylation activity lead to correlated levels of tumorigenicity potential. Proto-oncogenes can be strategically controlled by DNA mutations. The DNA mutations strongly influence roles for proto-oncogenes in cancer progression. Oncoproteins are a category of proteins that continue cancer progression.

Similar to mutations of proto-oncogenes, post-translational modifications influence the function of oncoproteins. PTMs can stimulate aberrant expression, function, and cellular drug resistance within a biological system. Two post-translational modifications have been linked to ZIC2. Phosphorylation is reported to aid in maintaining the role ZIC2 in the aforementioned protein complexes, investigated by Ishiguro et al.[15] Further work into the role of the DNA-PKcs exhibited how ZIC2 is modified. To identify the targeted site for phosphorylation, N-terminally truncated constructs were used for experimentation. Investigative evidence suggested that the site of phosphorylation of ZIC2 is Serine-200 by DNA-PKcs.

Two post translational modifications of interest for this study are sumoylation and ubiquitination. Lysine residues are essential components of both PTMs. Ubiquitination is a controlled process resulting in the attachment of a single Ub protein (mono-ubiquitination) or multiple Ub proteins (poly-ubiquitination). The three steps of

ubiquitination are activation, conjugation, and ligation. Activation of Ub is accomplished with catalysis of an E1 activation enzyme. Ub conjugation relies on the E2 conjugation enzyme for transfer to E3 ligating enzyme. The E3 ligase identifies the substrate and site of attachment and performs the ligation of Ub to protein substrate. The complete amino acid structure of ZIC2 provides a further depth of post-translational modifications (Figure 5).

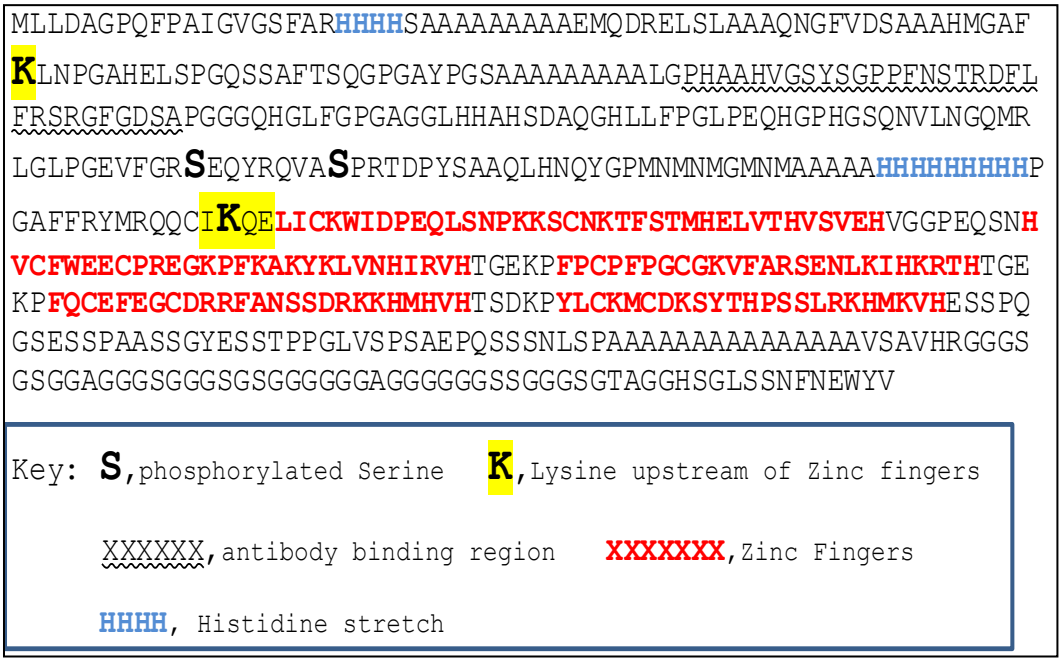


Figure 5. Amino acid sequence features of ZIC2.

There are two Lysine residues, which are located upstream of the zinc fingers at positions 61 and 253. Using a sumoylation prediction site, Lysine 253 was the only outcome for expected post-translational modification by sumoylation. Sumoylation is the process of the attachment of a small ubiquitin like modifiers, SUMO-1, SUMO-2, or SUMO-3.[51-52] A SUMO group is attached to its substrate through steps of enzymatic reactions by E1, E2, and E3 ligases.[52] The initial process begins with SUMO group

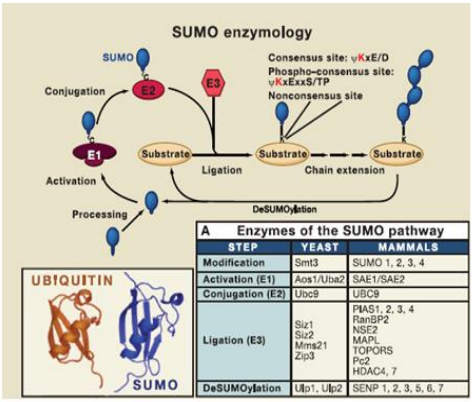
attachment to Sumoylation Activating enzyme (SAE, also known as, E1). [51]A transfer occurs between E1 and E2.[51] E2 is the conjugating enzyme, Ubc9.[51] E3 ligases are responsible for facilitating the sumoylation event and efficient attachment to the substrate.[51] A group of PIAS proteins comprise one of the E3 ligases used for that process(Figure 6).[52]

A

Predicted Sites

ID	Position	Peptide	Score	Cutoff	Type
sp|O95409|ZIC2_HU...	253	YMRQQCIKQELICKW	45.184	19.705	Sumoylation

B



A Enzymes of the SUMO pathway

STEP	YEAST	MAMMALS
Modification	Smt3	SUMO 1, 2, 3, 4
Activation (E1)	Aos1/Uba2	SAE1/SAE2
Conjugation (E2)	Ubc9	UBC9
Ligation (E3)	Siz1 Siz2 Mms21 Zip3	PIAS1, 2, 3, 4 RanBP2 NSE2 MAPL TOPORS Pc2 HDAC4, 7
DeSUMOylation	Ulp1, Ulp2	SENP 1, 2, 3, 5, 6, 7

Figure 6. The post-translational modification, Sumoylation: (A) Sumoylation prediction results for Lysine residues upstream of zinc fingers at position K61 and K253. (B) Sumoylation process of attachment to protein substrate.

Source: Adapted from Creton, S.; Jentsch, S. *Cell* **2010**, 143 (5), 848–848.

The consensus site for sumoylation is Ψ-K-X-D/E.[51-53] This sequence of four residues is comprised of a hydrophobic amino acid (Ψ), a lysine, any amino acid, and concludes with a glutamic or aspartic acid residue. The predicted site of sumoylation for ZIC2 is I-K-Q-E, as shown in Figure 7. This sequence is highly conserved amongst ZIC2 orthologs.

Chen et al. has investigated the sumoylation activity of ZIC3.[54] ZIC3 represses the gene promoter of cardiac α-actin. At the lysine 248 residue of ZIC3, a mutation to arginine was made and repressor activity was eliminated due to consensus site mutation for targeted sumoylation. The ubiquitination process flows into the 26s proteasome degradation pathway (Figure 7). De-ubiquitinating enzymes can reverse ubiquitination by cleaving Ub from the protein substrate.

A **B**

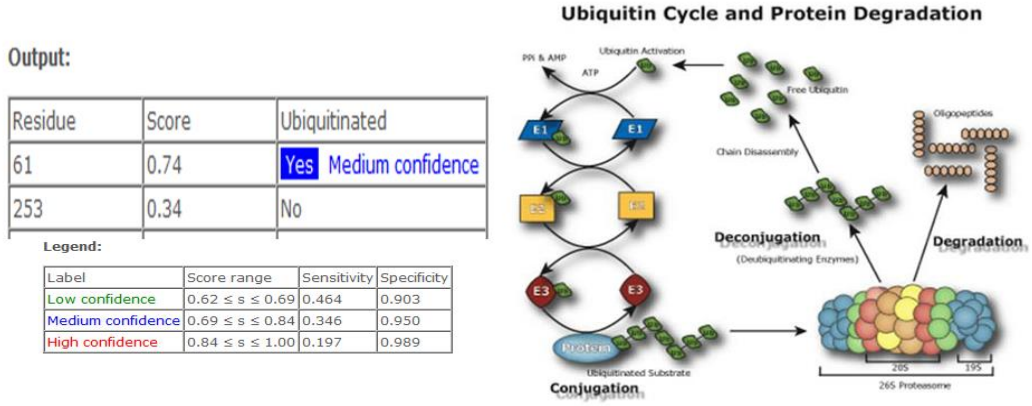


Figure 7. The post-translational modification, Ubiquitination: (A) Ubiquitination prediction results for Lysine residues upstream of zinc fingers at position K61 and K253. (B) Ubiquitin cycle and protein degradation process after substrate attachment.

Source: Adapted from BostonBiochem. http://www.bostonbiochem.com/products/ubiquitin (accessed Dec 28, 2015).

These modifications, in some instances, provide a necessary component to facilitate degradation.[51] The E3 ligase groups of proteins within eukaryotic systems are HECT, RING, and U-box proteins.[55] Within the RING group, RNF180 is an E3 ligase reported to interact with ZIC2.[56] The TCGA and cBioPortal information provides mRNA expression of RNF180 when compared to ZIC2 in benign and cancer samples of the PRAD dataset (Figure 8).[57] SPOP and SPOPL are additional E3 ligases with inverse

expression to ZIC2 in benign and cancer samples. SPOP was determined to be the most frequently mutated gene in 112 normal and prostate tumor samples.[58]

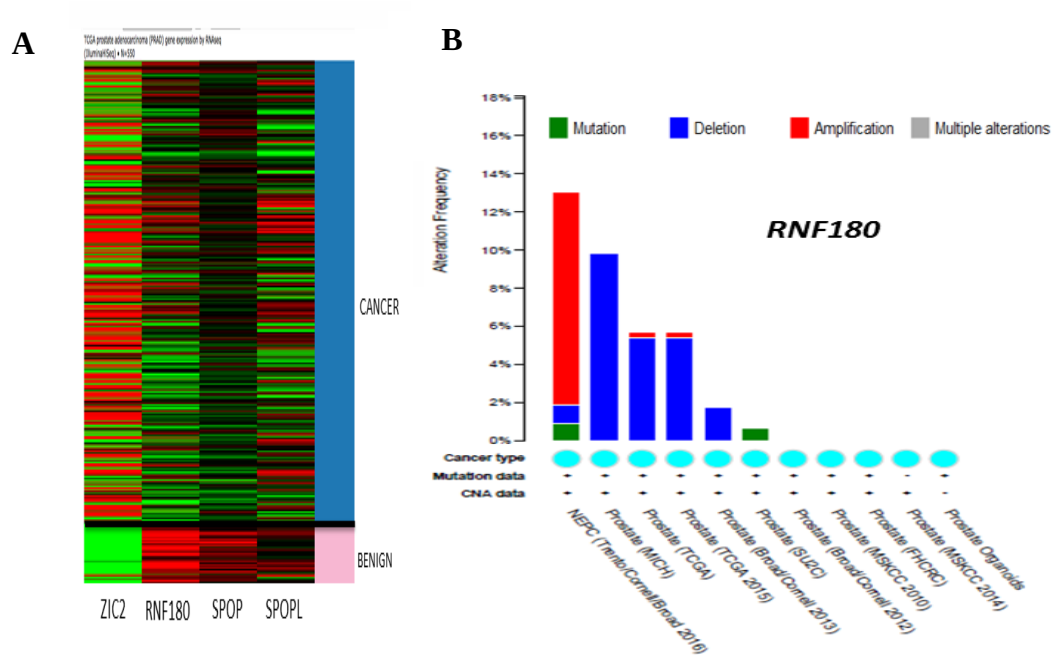


Figure 8. Bioinformatic analysis of RNF180 mRNA expression. (A) Data obtained from The Cancer Genome Atlas. (B) Data obtained from CBIOPortal.

SPOPL was incorporated in this analysis due to the heterodimeric formation with SPOP.[59] From the PRAD dataset, *RNF180* was reported to have the highest amplification frequency in Neuroendocrine Prostate Cancer (NEPC). *RNF180* was also reported to have deletions and mutations in a variety of Prostate cancer studies. In gastric cancer, RNF180 has been reported to be epigenetically silenced.[60-61] The epigenetic control could be problematic in the role of RNF180 in our PC-3 model in relation to aberrant expression of ZIC2.

It is proposed that ZIC2 is involved in prostate cancer progression and may be post-translationally modified. Here, the main question is how is it able to participate

during prostate cancer progression? In this study, we provide evidence in which the aberrant expression of ZIC2, that is not post-translationally modified and degraded, controls mitochondrial dysfunction in PC-3 cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 Mammalian Cell Culture

PC-3 prostate cancer cell line were obtained from ATCC and cultured at 37 °C, 5% CO2 in Dulbecco's modified Eagle's medium/F-12 mixed media (Gibco) supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin (Corning) and 10% fetal bovine serum (Atlanta Biologicals). Stable overexpressing cell lines were transfected with 2µg of Empty-MYC or ZIC2-MYC vector (PE and PZ notations) in OptiMEM media. After 72 hours, the cells were maintained in 1mg/ml or 1.5 mg/ml G418 (EMD Millipore).

3.2 CRISPR-*Cas9* Gene Editing

Targeted ZIC2 gene editing was achieved by transfecting 1x106 PC-3 cells in OptiMEM media using a 3 microliter to 1 microgram ratio of Fugene 6HD transfection reagent and the DNA 2.0 CRISPR-Cas9 plasmid pD1301-AD:154471 containing the 20-mer single guide RNA 5'-GAAGGCTCCCATGTGCGCGG-3'. This sequence is complementary to the coding nucleotides 161 to 180 in the first exon of the ZIC2 NCBI Reference Sequence: NM_007129.3. Cells were cultured for 72 hours under standard

conditions and then sorted by GFP fluorescence into single colonies by Fluorescence activated cell sorting (FACS) into a 96-well plate. Single cells were cultured for approximately two months to achieve confluency. Individual cell lines were then expanded and used for experimentation.

3.3 Proliferation

For cell growth experiments, $1x10^4$ cells were plated in triplicates of 6-well culture dishes. At the respective time courses, cells were obtained using 200µl of Trypsin/EDTA and diluted in 300µl of DMEM/F-12 media and counted using a Nexelcom cell counter.

3.4 Real Time Quantitative PCR

RNA was obtained from cells using the E.Z.N.A. RNA isolation kit (Omega Biotek) according to manufacturer's protocol. Complementary DNA was generated from 2µg of RNA and used for RT-qPCR. Taqman probes(Assay ID: Hs01120965_m1 and Gene Symbol: *TMPRSS2*, hCG401238; Assay ID: Hs01060665_g1and Gene Symbol: ACTB, hCG15971;Assay ID: Hs01103582_s1 and Gene Symbol: *JUN*, hCG1780282; Assay ID: Hs00233992_m1 and Gene Symbol: *MMP13*, hCG39650; Assay ID: Hs00158486_m1 and Gene Symbol: *KISS1*, hCG96217; Assay ID: Hs00171105_m1 and Gene Symbol: *CCNA1*, hCG32831; Assay ID: Hs02596866_g1 and Gene Symbol: *MT-CO3*, MYBPC3, SLC12A1; Assay ID: Hs00951307_m1 and Gene Symbol: *DKK3*, hCG23644) were used to amplify regions of interest according to the manufacturer's protocol.

3.5 Transient Transfections

To determine whether sumoylation both PTMs are required for ZIC2 stabilization, transient transfections in PC-3 cells was performed using the following plasmids: ZIC2-Myc-DDK, ZIC2- K61R-Myc-DDK, ZIC2-K253R-Myc-DDK, and ZIC2 K61R– K253R -Myc-DDK. An empty vector control was also used for transfection. After 48 hours, cell extracts were used for experimental analysis.

3.6 Migration and Invasion

Collagen coated 8.0μm transwell inserts were assembled into a 24-well plate and cell deposits of $3x10^4$ were allowed to migrate for 5 hours. For invasion assays, $1x10^5$ cells were allowed to invade 50μl of Matrigel (Corning) concentrated at 250μg/ml in 8.0μM transwell inserts. Cellular invasion was assessed after 24 hours with a 5% complete media chemoattractant. For both assays, cells were suspended in serum-free media. Fixed cells were stained with Nucblue (Life Technologies) and imaged for counting.

CHAPTER 4

RESULTS

To address our hypothesis we have firstly evaluated whether or not ZIC2 is expressed at steady state levels within a select group of Prostate cancer cell lines. The predicted molecular weight of ZIC2 is 55kDa. In order to establish *in vitro* models of ZIC2 expression on prostate cancer, we also probed for ZIC2 expression in various human prostate cancer cell lines (Figure 9). We chose a sample of cells lines representing both ethnicity and Androgen receptor (AR) status. E006AA is a primary AR positive, African American prostate cell line and E006AA-hT is a highly tumorigenic subline.[62] LNCaP is also an AR positive cell line of Caucasian origin.[63] DU145 represents an AR negative cell line while PC-3 represents an AR negative and highly aggressive cell line model.[64-65] All prostate cancer cells examined by immunoblot express a common ZIC2 band that runs just above 65 kDa. Androgen receptor (AR) positive cell lines (E006AA, E006AA-hT, and LNCaP) also express a lower ZIC2 band just below 65 kDa.

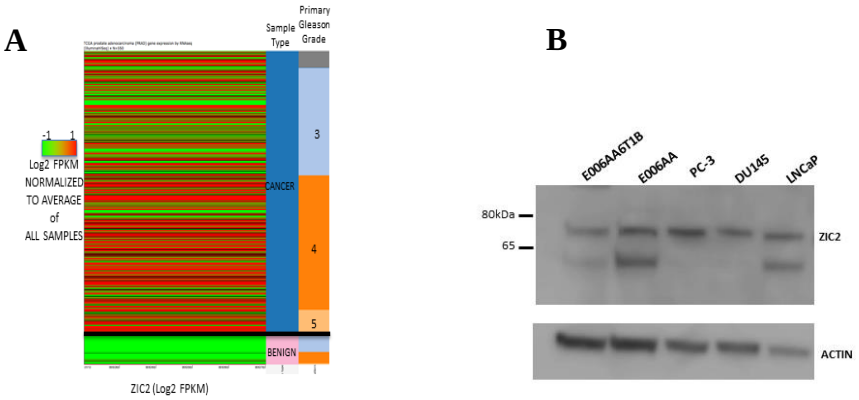


Figure 9. ZIC2 is expressed in human prostate tissue and prostate cancer cell lines. **(**A) ZIC2 mRNA expression analysis in cancer (blue) and benign samples (pink). Prostate adenocarcinoma dataset from the Cancer Genome Atlas using Illumina HiSeq throughput, N=550. (B) ZIC2 is expressed in several Prostate cancer cell lines.

4.1 Genome Edited ZIC2 Knockdown with CRISPR-*Cas9*

In order to determine if ZIC2 contributes to aggressive prostate cancer cell growth, we subjected the PC-3 cell line model to CRISPR-Cas9 gene editing of ZIC2. CRISPR-Cas9 gene editing was successfully performed with the single guide RNA 118 that spans coding nucleotides 161 to 180 in the first exon of the ZIC2 NCBI Reference Sequence: NM_007129. Clonal cell lines resulting from single cell sorting of edited cells were evaluated for ZIC2 alterations at both the DNA sequence and protein expression levels. Three reads from each allele revealed 1 and 2 bp deletions at position 163 of the ZIC2 mRNA NCBI Reference Sequence: NM_007129.3 (Figure 10). No parental reads were recovered from this edited cell line. The karyotype of the parental PC-3 cell line has previously been reported by multiple laboratories as being diploid at Chromosome 13q32.3, the genomic location of ZIC2.

Figure 10. CRISPR-*Cas9* Loci of sgRNA targeting ZIC2. Nucleotide (upper) and amino acid (lower) alignments of the ZIC2 coding region mutated by CRISPR/Cas9 gene editing in PC-3. The black box indicates the codon "GCG" coding for Alanine in the parental/ "wild type" ZIC2. One edited allele contains a single "G" nucleotide deletion at coding nucleotide 163 resulting in a frameshift mutation of Alanine (A) to Arginine (R) at amino acid position 55. The other allele contains a "GC" dinucleotide deletion at coding nucleotides 163 and 164 resulting in a frameshift mutation of Methionine to Glycine at amino acid position 57.

Therefore, we hypothesize that we have created a homozygous null allele for the full length ZIC2 protein in the PC-3 cell line, hereafter referred to as PC-3 ZIC2 -/-. One clone (shown in culture in Figure 11), that we named according to the Human Genome Variation Society (HGVS) guidelines as PC-3 NM_007129.3(ZIC2):c.[163delG;163_164delGC], contained mutations at two alleles.[2] These alleles were confirmed in this cell line by identification of at least three identical Sanger sequencing reads from cloned genomic DNA PCR products spanning the first exon of ZIC2.

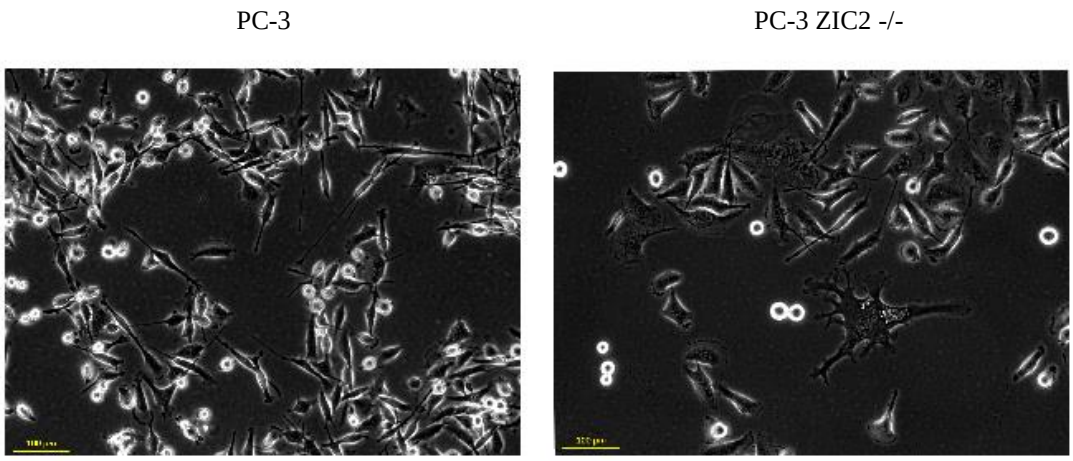


Figure 11. Phenotype of wild-type and CRISPR-*Cas9* mutant cells. Microscopic brightfield images of PC-3 parental cells and PC-3 ZIC2 -/- cells.

Western blot analysis confirmed that ZIC2 protein levels in PC-3 ZIC2 -/- cells were indeed altered when compared to the PC3 ZIC2 parental cell line using the rabbit monoclonal antibody (Figure 12).

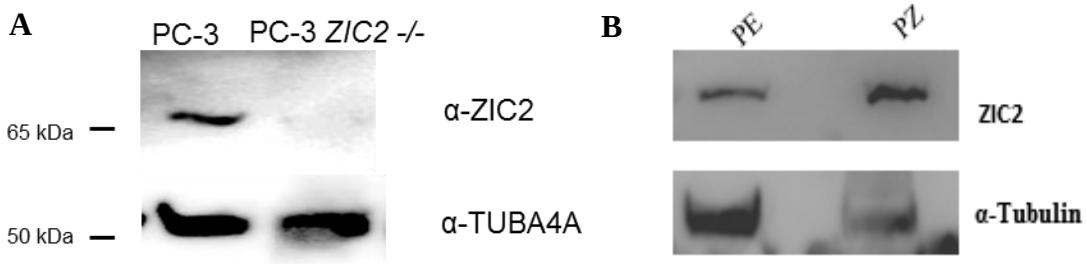


Figure 12. Western blot of ZIC2 protein expression in PC-3 parental and PC3 ZIC2 -/- edited cells. WT and mutant immunoblot analysis of ZIC2 expression. (A) Differential expression among mutations after CRISPR Cas9 editing in PC-3 cells. (B) Stable overexpression in PC-3 +ZIC2 (PZ). E denotes empty vector control (PE).

4.2 ZIC2 Knockdown Impairs Cellular Proliferation

To initially characterize the phenotype of the PC-3 ZIC2 -/- cells, we first performed cell proliferation assays in the form of growth curve analyses. As early as day

five, ZIC2 -/- cells exhibited a significant decrease in cell growth when compared to the parental PC-3 cells and this difference expanded to a nearly 5-fold decrease by day seven (Figure 13). This data provides the first functional evidence supporting a role for ZIC2 in the promotion of prostate cancer cell growth.

To assess the influence of mutagenesis, we evaluated cellular growth in the PC-3 ZIC2 -/- cells as well as the stably overexpressed cells. PC-3 ZIC2 -/- cells grew approximately 5 times slower than the PC-3 parental cells within one week. The doubling time of PC-3 cells has an effect on the growth throughout the time course which influences the significant differences between PC-3 and PC-3 ZIC2 -/- cells at day 5. Furthermore, in stably overexpressed PZ cells, we observed PZ cells growing twice as fast as the empty vector control. This data provides evidence of ZIC2 influencing cellular proliferation in a prostate cancer model. The growth rate differences amongst the PC-3 WT and PE are distinguishable by the G418 stably selected PE cells. G418 resistance has been linked to metabolic kinetics of the enzyme-ADP complex.[66] The cellular metabolism and growth rates are reduced in the presence of G418.[67]

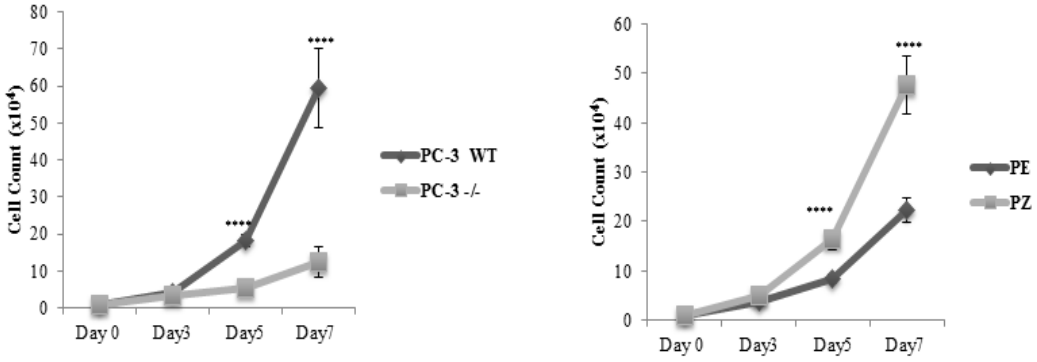


Figure 13. Proliferation growth curves for mutant cell types. Growth analysis of PC-3 cells exhibit significant growth differences with the knockdown cells. PC-3 cells exhibit significant growth differences with the knockdown cells showing slower while the PZ cells grow faster than the empty vector control.

4.3 ZIC2 Knockdown Hinders S Phase Entry of Cell Cycle

After detailed evaluation of PC-3 proliferation, we explored the cell cycle phase that supports the cell growth differences PC-3 ZIC2 -/- cells demonstrates a greater phase within G0/G1compared to PC-3 WT. This data supports the proliferation analysis due to the absence of cell cycle arrest. Our results indicate a potential role for ZIC2 in the control of cell cycle but not a direct arrest. The overexpressed model did not have a significant observation in cell cycle phases associated with replication and division although there was an observation of increased cell growth. After evaluation of cell proliferation, we performed cell cycle analysis of the

PC-3 ZIC2 -/- cells by flow cytometry of propidium iodide (PI) stained cells (100K). When compared to parental PC-3 cells, we found an increased percentage of ZIC2 -/- cells in G1 (57% in PC-3 ZIC2 -/- ; 39% in PC-3), indicating a sizeable G1 arrest in the mutant cells (Figure 14).

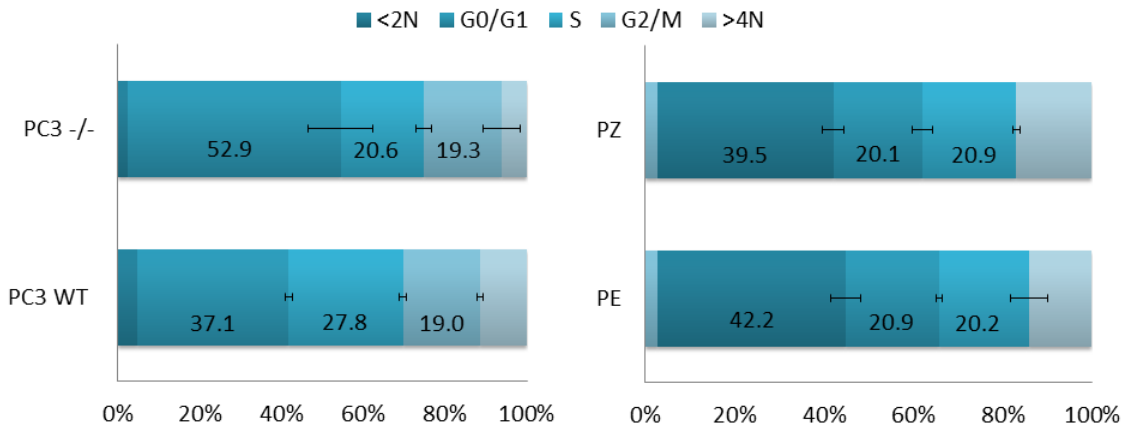


Figure 14. Cell cycle analysis of PC-3 cells Cell cycle analysis of PC3 -/- cells and PZ cells. Flow cytometry analysis of cells was measured using 100,000 events.

4.4 ZIC2 Deters Migration and Invasion in Mutant PC-3 Cells

More detailed investigations of functional analysis yielded information leading to the influence of ZIC2 in migration and invasion of PC-3 cells. To date there have not been studies relating ZIC2 to migration and invasion in a prostate cancer model (Figure 15). Due to our evidence of ZIC2 knockdown severely decreasing cellular proliferation, we also wanted to determine the transwell migration and Matrigel invasion potential of mutant and stably overexpressed PC-3 cells. PC-3 ZIC2 -/- cells demonstrated slower migration while the PZ cells did not significantly have altered migratory effects. Matrigel invasion of PC-3 ZIC2 -/- cells also demonstrate decreased invasiveness will PZ cells exhibited a significant increase in invasion. These results suggest a role for ZIC2 in prostate cancer cell migration and invasion. This is significant due to the overall classification of PC-3 cells traditionally representing an aggressive prostate cancer phenotype.

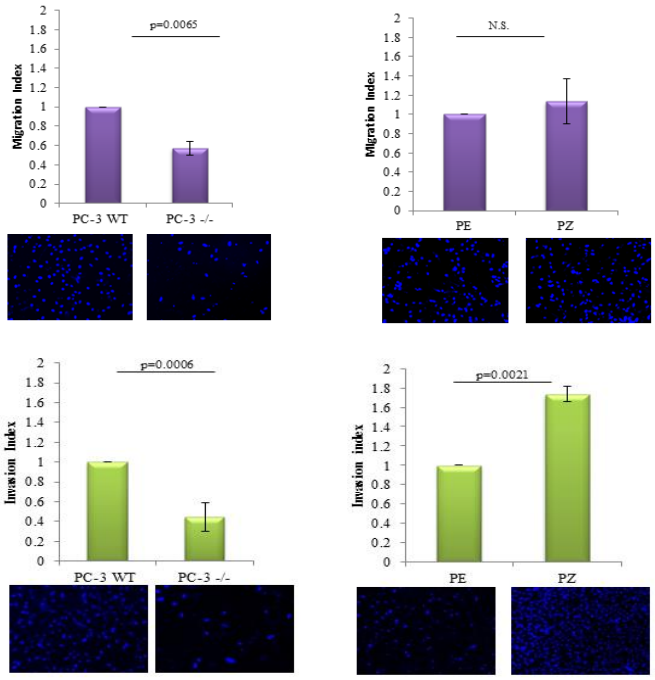


Figure 15. ZIC2 is required for proper migration and invasion. Migration and invasion cell counts were normalized to the WT and empty-vector controls. Nuclei images below each graph are representative from an analysis of N=3 independent experiments. Error bars are represented as ±SEM. p-Value notation is denoted as N.S. =not significant; *=$p<0.05$; **= $p\leq0.01$; ***=$p\leq0.001$

4.5 RNA-Seq of PC-3 ZIC2 -/- Cells

ZIC genes are most closely related to the GLI family of transcription factors that are downstream effectors of the Hedgehog signaling pathway. Therefore, we reasoned that ZIC2 may in part play a role in promoting the aggressive phenotype of PC-3 by transcriptional regulation. In order to reveal the transcriptome changes in PC-3 ZIC2 -/-, we performed RNASeq on total RNA from PC-3 ZIC2 -/- and PC-3 parental cells. Sequencing of rRNA depleted and random-primed cDNA from PC-3 parental cells yielded 19,938,569 uniquely mapped reads, whereas the PC-3 ZIC2 -/- cells yielded 40,448,543 uniquely mapped reads. In total, 734 differentially expressed genes (DEGs)

were identified using cuffdiff with a threshold of p<.05 and a greater than 2-fold change in gene expression. We report a short list of identified DEGs in Table 1.

Table 1. RNA Sequencing Results of Differentially Expressed Genes in PC-3 ZIC2 -/- Cells

Gene	Gene Name	Log_2 fold change	p-Value
MMP13	Matrix Metallopeptidase 13	-6.06874	0.00035
FOS	FBJ Murine Osteosarcoma Viral Oncogene Homolog	-5.10708	0.00115
CCNA1	Cyclin A1	-4.28057	0.03835
CYP1B1	Cytochrome P450, Family 1, Subfamily B, Polypeptide 1	-3.53682	0.01605
CYP11A1	Cytochrome P450, Family 11, Subfamily A, Polypeptide 1	-3.35387	0.04485
SNAI1	Snail Family Zinc Finger 1	-3.23629	0.0116
PIM3	Pim-3 Proto-Oncogene, Serine/Threonine Kinase	-2.07805	0.0042
JUN	Jun Proto-Oncogene	-1.92282	0.0039
HSP90B1	Heat Shock Protein 90kDa Beta (Grp94), Member 1	-1.77878	0.03595
CDKN1B	Cyclin-Dependent Kinase Inhibitor 1B (P27, Kip1)	-1.76498	0.0222
CDKN1C	Cyclin-Dependent Kinase Inhibitor 1C (P57, Kip2	-1.75779	0.0389
MT-CO3	Mitochondrially Encoded Cytochrome C Oxidase III	1.56683	0.02175
PRMT6	Protein Arginine Methyltransferase 6	2.04488	0.04975
TMPRSS2	Transmembrane Protease, Serine 2	2.27752	0.0141
ABCG1	ATP-Binding Cassette, Sub-Family G (WHITE), Member 1	2.77831	0.00835
KISS1	KiSS-1 Metastasis-Suppressor	2.84573	0.0174
UCHL1	Ubiquitin Carboxyl-Terminal Esterase L1 (Ubiquitin Thiolesterase)	3.18991	0.01475
DKK3	Dickkopf WNT Signaling pathway inhibitor 3	4.33646	0.0019
IL6	Interleukin 6	4.47851	0.0035

We verified a subset of these DEGs by quantitative reverse transcription polymerase chain reaction (qRT-PCR), as shown in Figure 16. To validate genes related to proliferation, migration and invasion we chose *MMP13*, *CCNA1*, *JUN*, *KISS1* and *DKK3* for analysis. Our results confirmed that pro-growth genes such as *CCNA1*, *JUN* and *MMP13* were down-regulated in PC-3 ZIC2 -/- cells and that genes associated with cell differentiation or tumor suppressor activity including *KISS1*, *DKK3* and *TMPRSS2* were upregulated in PC-3 ZIC2 -/- cells. These results further support a role for ZIC2 in promoting prostate cancer cell growth.

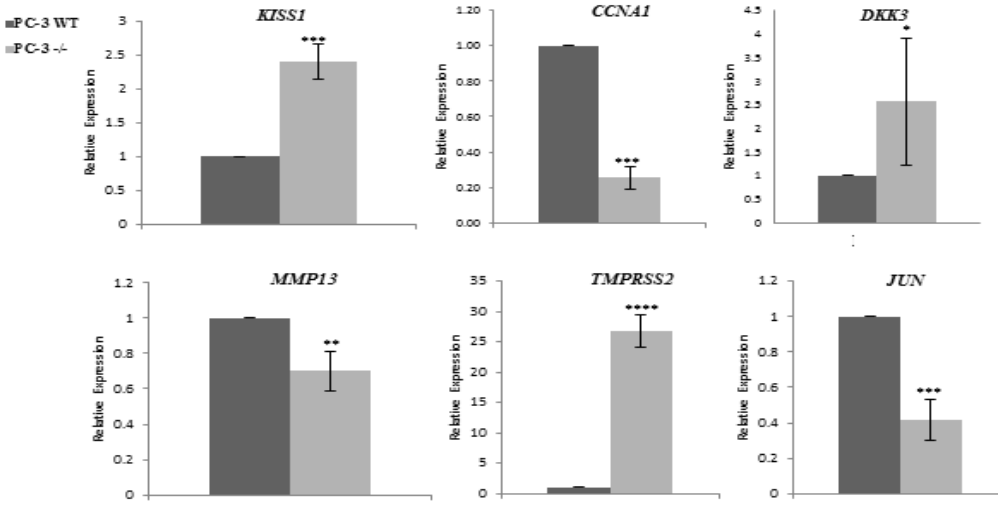


Figure 16. qRTPCR Gene expression confirmation from RNA sequencing results of several genes linked to up regulation and downregulation in ZIC2 knockdown PC-3 cells. RNA sequencing revealed several genes associated with down/up regulation.

4.6 Cancer Cell Metabolism Genes

Warburg established that even in the presence of oxygen, cancer cells display an increase in glycolysis and lactic acid production along with reduced levels of mitochondrial oxidative phosphorylation. We present RNA sequencing results of differentially expressed genes linked to energy metabolism in Table 2. Consistent with PC-3 performing high rates of glycolysis, the sugar/H+ symporter *SLC2A13*, is expressed at high levels in PC-3 and significantly decreases in PC-3 ZIC2 -/- cells. Although not significant, key genes involved in glycolysis including *PGK1*, *ENO1*, *ENO2* and *ENO3* are also downregulated in the PC-3 ZIC2 -/- cells. Conversely, genes involved in mitochondrial oxidative phosphorylation, such as the mitochondrial *MT-CO3* are expressed at significantly higher levels in the PC-3 ZIC2 -/- cells. We confirmed the *MT-CO3* expression by qRT-PCR (Figure 17). Other mitochondrial genes upregulated in PC3

ZIC2 -/- cells include *MT-ATP6* and *MT-ATP8* suggesting increased mitochondrial ATP production. The cancer stem cell marker, CD44, has recently been shown to promote glycolysis in PC-3 cells.[68] PC3 ZIC2 -/- cells show significantly decreased *CD44* expression when compared to PC-3 parental cells.

Table 2. RNA Sequencing Results of Differentially Expressed Genes Linked to Energy Metabolism in PC-3 ZIC2 -/- Cells

Gene	**Gene Name**	**Log_2 Fold Change**	**p-Value**
SLC2A13	Solute Carrier Family 2 Member 13	-2.684	0.015
CD44	CD44 molecule (Indian blood group)	-1.997	0.0112
ENO2	Enolase 2	-1.372	0.660
PGK1	Phosphoglycerate Kinase 1	-1.236	0.251
ENO3	Enolase 3	-1.180	0.250
ENO1	Enolase 1	-0.802	0.393
MT-ATP6	Mitochondrially Encoded ATP Synthase 6	0.978	0.657
MT-ATP8	Mitochondrially Encoded ATP Synthase 8	1.248	0.733
MT-CO3	Mitochondrially Encoded Cytochrome C Oxidase 3	1.567	0.022
MT-CYB	Mitochondrially Encoded Cytochrome B	1.568	0.073

Additional evidence indicating increased metabolic activity in the mitochondria of PC-3 ZIC2 -/- cells was shown by an unexpected increase in the metabolic activity as measured indirectly by increased MTS(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) cell viability

assay.[69] PC-3 ZIC2 -/- cells display significantly higher MTS assay levels as indicated by the increased relative fluorescence when compared to PC-3 parental cells.

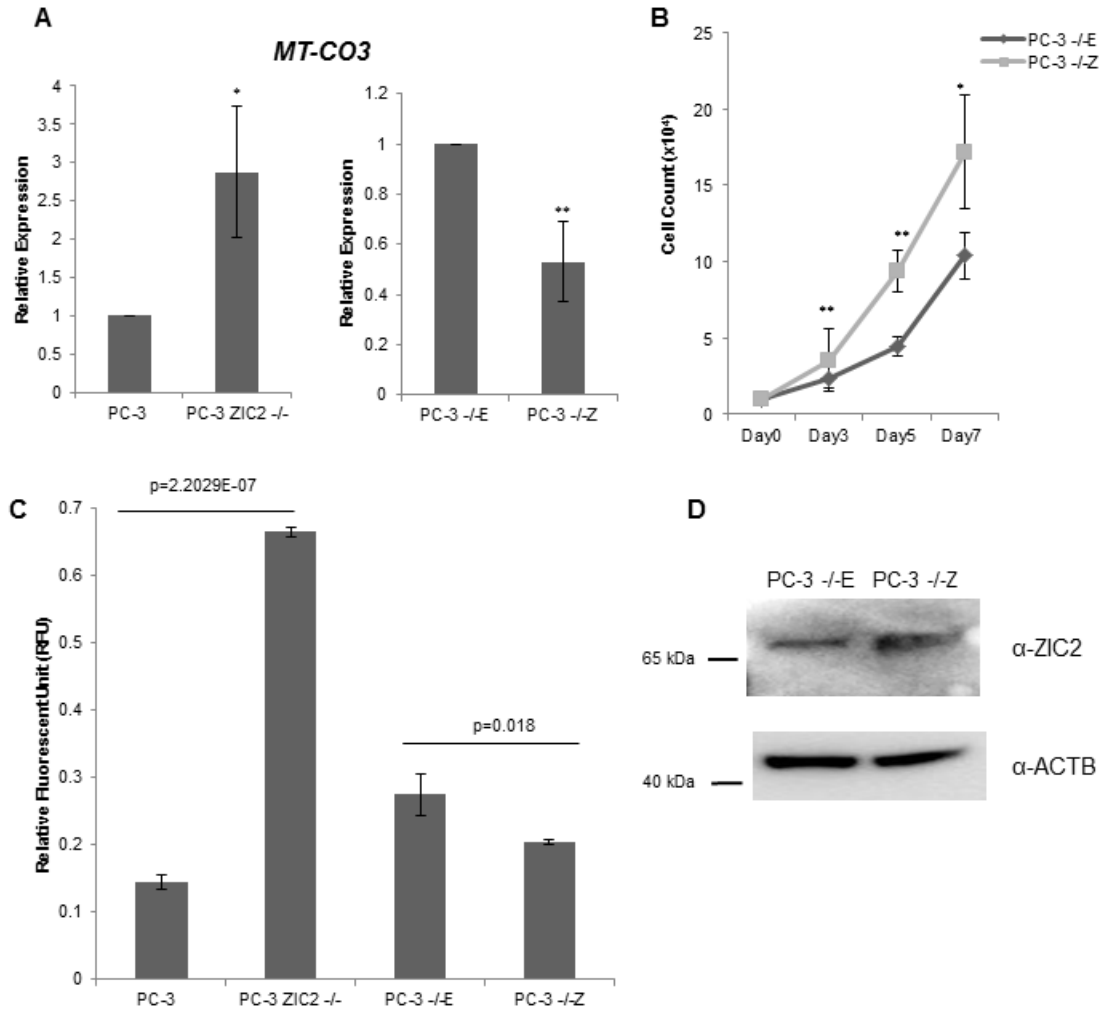


Figure 17. Metabolic characterization of PC-3 ZIC2 -/- and ZIC2 rescued cells. **(**A) Up-regulation of *MT-CO3* in ZIC2 knockdown is recovered with stable re-expression of ZIC2. (B) Cell proliferation of rescued phenotype. -/-E is abbreviated for mutant transfected with empty control and -/-Z is abbreviated for mutant transfected with ZIC2. This graph represents rescued growth of PC3 ZIC2 -/- cell with ZIC2 insertion.(C) ZIC2 indirectly affects cellular metabolism switch via initiation of oxidative phosphorylation.(D) Immunoblot confirmation of ZIC2 rescue in PC-3 cells.

4.7 Rescue of ZIC2 Edited Cells

In order to provide evidence that the CRISPR-*Cas9* gene editing in PC-3 cells was specific for ZIC2 and to determine whether the phenotypes induced in the edited cells could be rescued, we stably re-expressed a full length ZIC2 ORF in the PC-3 ZIC2 -/- cells. We measured cell proliferation by growth curve analysis and found that increased proliferation in these cells was recovered. *MT-CO3* mRNA levels were measured by qRT-PCR and shown to significantly decrease in the ZIC2 rescued cell line. Finally, in order to test whether energy metabolism had been affected by ZIC2 re-expression, the MTS assay was performed on the rescued cells. The results shown in Figure 17 indicate the MTS assay product, formazan, was also significantly reduced in the presence of increased ZIC2 protein expression. We measured ZIC2 protein levels by immunoblot analysis and confirmed increased expression of ZIC2 in the rescued cell lines. In combination, our results from the ZIC2 rescued cell line indicate a reversal towards the original PC-3 cancer cell phenotype.

4.8 Ingenuity Pathway Analysis of Potential Roles for ZIC2

4.8.1 Migration and Invasion

RNA sequencing data was the input for IPA to generate specific models implicating ZIC2 in cell cycle progression, metabolism, and metastatic potential. The canonical pathway addressing cellular metastasis overlapping with significant genes from our dataset reveals alternating pathways for impacts in cell migration. One pathway implicates Wnt signaling and downstream targets: PKA, β-catenin, TCF/LEF to downregulate expression of MMPs in PC-3 ZIC2 -/- cells. In contrast, the second

pathway abbreviates migration and invasion through the ERK pathway and angiogenic regulation by the FOS and *JUN* dimerization and VEGF (Figure 18).

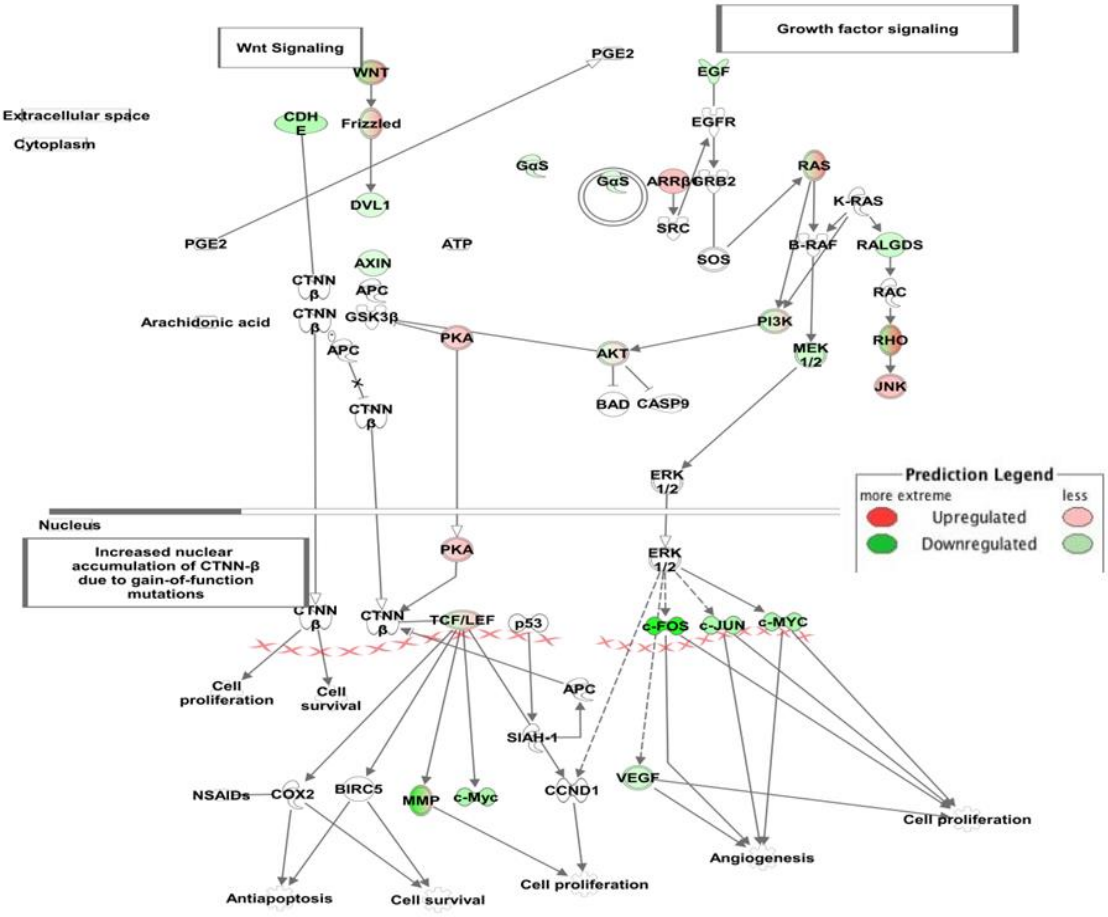


Figure 18. Metastatic pathway implicating expression of several genes up and downregulated as a result of ZIC2 knockdown.

4.8.2 Cell Cycle Analysis

Cellular proliferation and division is controlled in a very detailed manner. Figure 19 suggests the multiple down and upregulated genes involved in this very specific

component of the cell. G1/S phase transition is reported to be affected by p27.

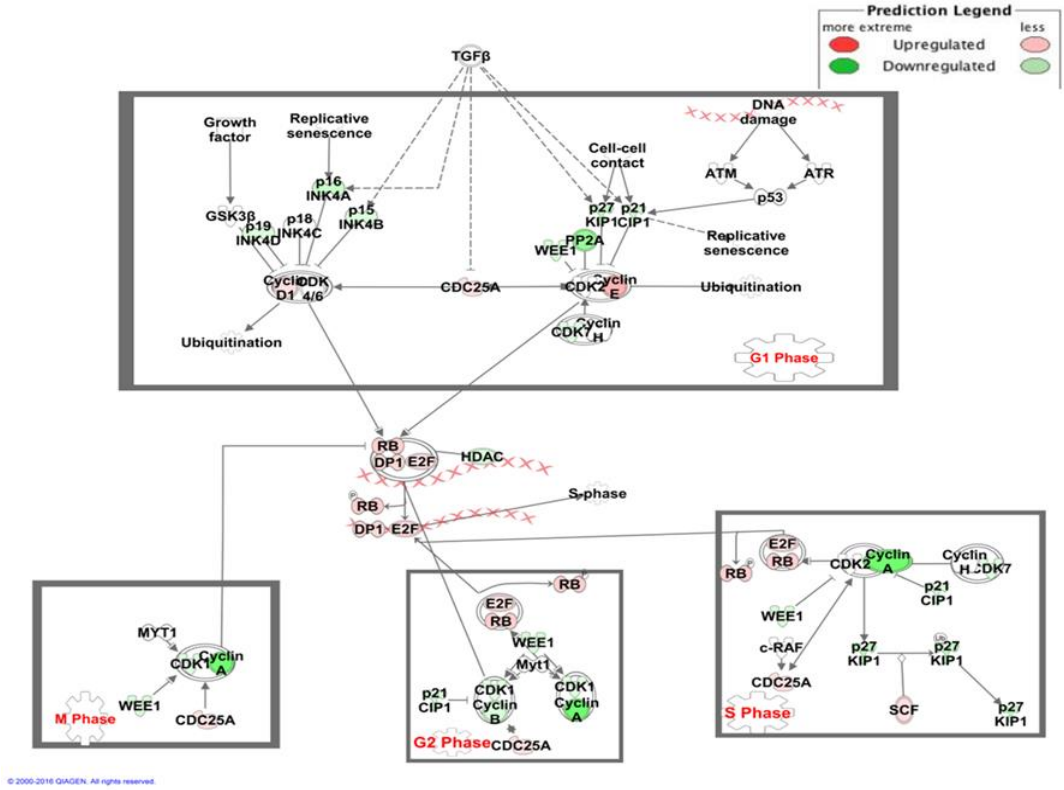


Figure 19. Cell cycle pathway identifying altered gene expression from RNAseq results in every phase.

4.8.3 Mitochondrion Metabolism

Oxidative phosphorylation is a metabolic process used to generate ATP molecule utilized for cellular energy. Cancer cells utilize the expensive Warburg effect rendering the mitochondria inactive. The importance of ZIC2 regulating a switch from glycolysis to increased oxidative phosphorylation has been implicated by upregulating *MT-CO3* (Figure 20). *MT-CO3* directly interacts with *HIF1-α* and *SLC25A13*, reported by IPA analysis. *HIF1-α* is reported in several studies to promote tumor progression through ROS generation.[70-72]

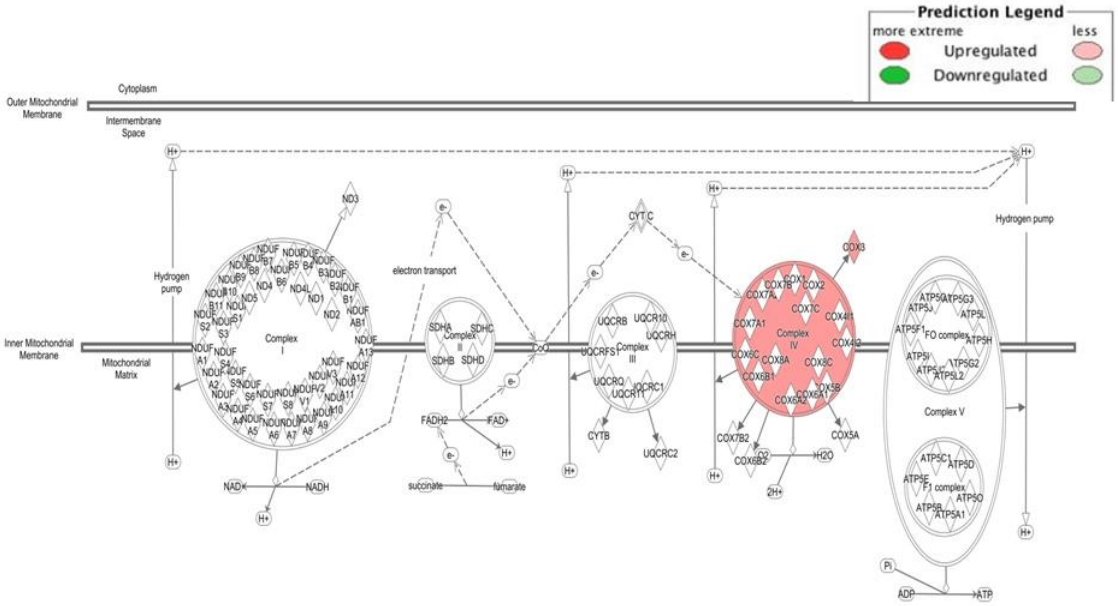


Figure 20. Oxidative phosphorylation pathway to highlight upregulation of *MT-CO3*.

4.9 Re-introduction of ZIC2 in Mutant PC-3 Cells

In an effort to determine rescued phenotype of ZIC2 in PC-3 ZIC2 -/- cells, we transfected ZIC2 vector and made stable with selection. We have successfully recovered the cellular growth showing significance within the 7 day time course. ZIC2 -/-Z has an approximate cell growth approximately 3 times greater than -/-E control. The cell growth of ZIC2 -/-Z is shown to be significant. We also demonstrate real-time PCR analysis of gene targets (indirect and direct) with ZIC2-/- and ZIC2 -/-Z. Protein expression also eliminates the any off-target effects and supports restoration of ZIC2 as a tumor promoter. Our pathway analyses promote specific roles for ZIC2 in multiple cellular processes.

4.10 Post-translational Investigation of ZIC2 Protein

Protein turnover is essential for homeostatic regulation of intracellular proteins. Generation and degradation is controlled by post-translational modifications. Mostly, protein degradation is controlled by ubiquitin mediated proteasome pathway. This pathway allows for mono- or poly- ubiquitination ligation to substrate by a class of enzymes, E3 ligases. Sumoylation is an additional post translational modification that has been reported to assist in protein stabilization and subsequent ubiquitin attachment. RNF180 is an E3 ligase with a molecular weight of 68 kDa. To date the predicted ubiquitination site within ZIC2 has not been identified. We hypothesize that the oncoprotein ZIC2 is targeted for degradation by the ubiquitin proteasome pathway via interaction with RNF180. To determine the necessity for the lysine residues upstream of the zinc fingers in the ZIC2 sequence, we sought to transiently overexpress ZIC2 with site-directed mutagenesis (Figure 21).

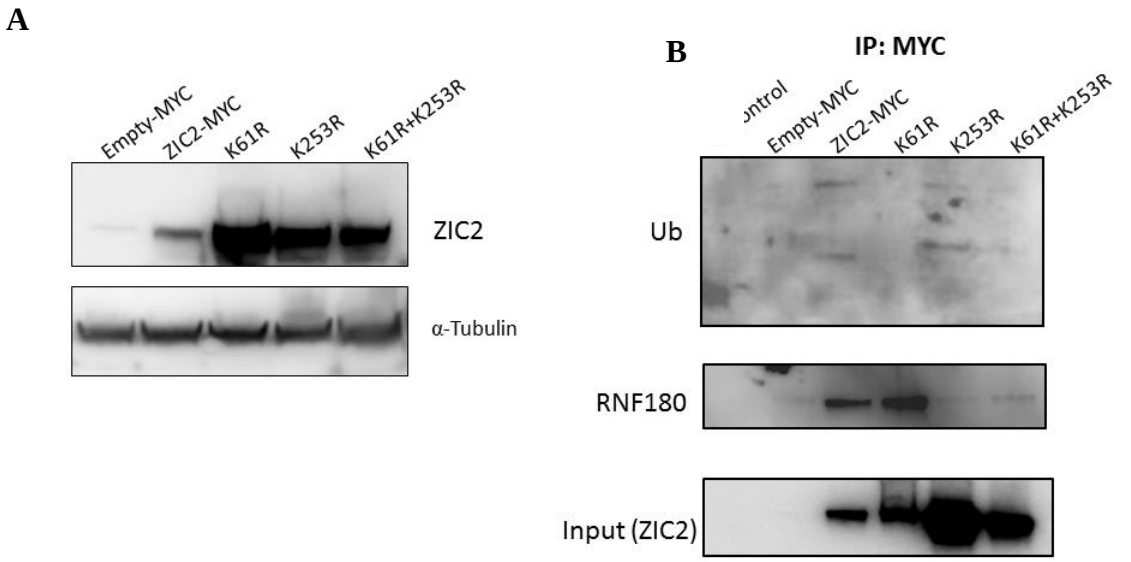


Figure 21. (A) Transient transfection of ZIC2 K61R, K253R, and K61R+K253R. (B) Immunoprecipitation of ZIC2 K61R, K253R, and K61R+K253R interacting with Ub and RNF180.

We have examined the ZIC2 protein expression in PC-3 cells overexpressing ZIC2 K-R plasmids. It is exhibited that ZIC2 is not degraded when the K61R position is compromised. Sumoylation at the K253R position is abolished however ubiquitination is a viable PTM. Altering both lysine residues aids in the stabilization or increased expression because both positions are compromised. ZIC2 has a role as a transcriptional regulator in the nucleus. In order to determine the nuclear localization of K-R mutagenesis, we assessed the localization by immunofluorescence (Figure 22). The nuclear localization was not altered. Immunoprecipitation of ZIC2 overexpression vectors with site-directed mutagenesis was achieved by precipitation of MYC-DDK tagged ZIC2 (WT, K61, K253, and K61+K253) and immunoblotted with Ub and RNF180 antibodies. Ubiquitin interaction was observed in the ZIC2 WT overexpression and K253R. This result concludes the K61 residue as the ubiquitination site. Immunoblotting with RNF180

exposed a different position necessary for interaction with the E3 ligase. The K253 position compromised an interaction with RNF180 while the ZIC2 WT and K61 displayed an interaction with RNF180.

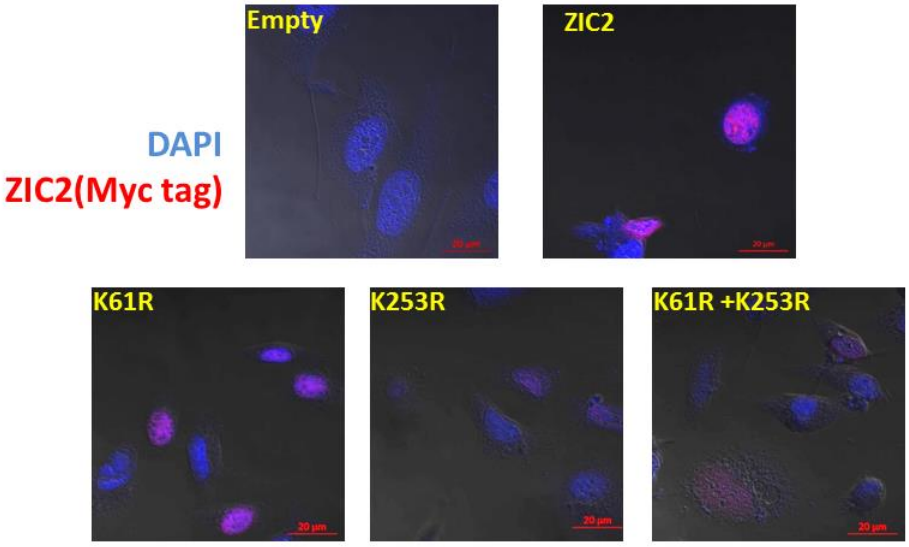


Figure 22. Immunofluorescence analysis of K-R mutation does not inhibit nuclear localization of ZIC2-Myc. Modifications of Lysine residues upstream of zinc fingers show that localization of ZIC2 is not affected by K-R but PTM stabilizes ZIC2 expression in K61R.

CHAPTER 5

CONCLUSION

This study highlights aberrant expression of ZIC2 in prostate cancer that contributes to the worsened progression. We have provided evidence of ZIC2 being aberrantly expressed in prostate cancer samples of the cancer genome atlas database. This gene expression data along with an initial study of ZIC2 by Tomlins et al. thrusted us to elucidate the role for ZIC2 in prostate cancer.

To establish *in vitro* models for investigating *ZIC2* function in prostate cancer, we identified ZIC2 expression by immunoblot in multiple prostate cancer cell lines. ZIC2 protein was found to be expressed in all prostate cancer cell lines examined. One significant observation from our data is the detection of differing patterns of ZIC2 protein bands between the AR positive and AR negative cell lines. In all cell lines, we observe a protein with a molecular weight just above 65kDa. This difference in molecular weight, from the predicted 55kDa for ZIC2, suggests the occurrence of post-translational modifications. Previous studies have identified phosphorylation of ZIC2 at amino acid S191 and S200, but an altered protein migration patterns was not observed in those studies. From our identification of ZIC2 expression in prostate cancer cell lines, we chose PC-3 cells for further study due to their aggressive cancer phenotype including their similarity to lethal prostate cancer.[73] To evaluate ZIC2 loss of function in prostate cancer

cell lines, we employed CRISPR-*Cas9* gene editing of ZIC2 in PC-3. PC-3 *ZIC2* -/- cells show altered expression of ZIC2 and significantly decreased proliferation rates when compared to the PC-3 parental cells. Additionally, siRNA mediated ZIC2 knockdown induced cell apoptosis while ZIC2 overexpression enhanced proliferation in pancreatic cancer cells.[35] The reduced proliferation rate in ZIC2 -/- cells that we report here also suggest a possible increase in apoptosis or senescence in prostate cancer when ZIC2 levels are altered.

We also present data indicating that ZIC2 promotes metastasis in lethal prostate cancer. Here, we report that migration and invasion capabilities of PC-3 ZIC2 -/- cells were significantly reduced when compared to PC-3 parental cells. These assays are used as *in vitro* models to measure the metastatic potential of cancer cells, a hallmark of aggressive lethal prostate cancer. In a completely different context, ZIC2 was recently shown to control the migration of neurons within the developing mouse forebrain.[33] This observation, along with our findings strengthens the connection between ZIC2 expression and cell migration.

Furthermore we also highlight the migration and invasion potential of PC-3 ZIC2 -/- cells. The migration and invasion was significantly deterred by ZIC2 knockdown. However, PZ cells did not significantly have the migration potential greater than the PE control. This could be a result of PZ cells undergoing cellular division in opposition to migration. We did see the invasion potential significantly increase in the PZ cells. ZIC2 was reported to control the migration of neurons within the developing mouse

forebrain.[33] This along with our studies contributes the role ZIC2 may have in cell migration.

We expected to observe an undersized S phase due to the significant increase of the cell proliferation of PZ cells. The distinct changes in phases from one to the next was not completely captured due to the hypothesized speed of a complete cell cycle in PZ cells progressing double or triple replications and divisions compared to a single division of the wild-type. Traditionally, cyclin dependent kinase inhibitors have been well studied as negative regulators of the cell cycle. *CDKN1B*, specifically, has a specific role in G1/S phase transition. It is hypothesized that the normal activity of *CDKN1B* assists the cell into S phase entry. Our overlapping analysis from IPA displays *CDKN1B* involvement however it is necessary to understand the Restriction point within cancer cells is dysregulated. Thus, proceeding into S phase is not rescued by the Restriction point due to S phase promoting factor. *CCNA1* which is also significantly downregulated supports a role for the PC-3 ZIC2 -/- cell to proceed through G1 but replication is delayed to the downregulation of *CCNA1*. Our proliferation growth curves do not support immediate programmed cell death but possibly senescent activity for the cell to remain proliferative.

We have utilized RNA-Seq to evaluate several genetic alterations in the oxidative phosphorylation pathway. One gene in particular, *MT-CO3*, was significantly upregulated in the ZIC2 knockdown model. *MT-CO3* operates in Complex IV of the oxidative phosphorylation pathway. We highlight the metabolic switch in PC-3 cells using the MTS assay that utilizes colorimetric changes in the presence of active mitochondria. Our

results thus far promote ZIC2 as an oncoprotein. We have noticed a significant decrease in the cell proliferation of the ZIC2 knockdown cells compared to the wild-type PC-3 cells. Introduction of ZIC2 as proof of concept rescue implicated contrasting results and allowed a metabolic switch identified by the MTS assy. Although the mechanism of how aberrant expression of ZIC2 suppresses oxidative phosphorylation remains to be elucidated, we provide evidence that ZIC2 expression aids in the control of mitochondrial dysfunction in PC-3 cells.

Our data revealed an unexpected significant increase in metabolic activity in the slower growing PC-3 ZIC2 -/- cells as measured by the MTS assay. We suggest this increase in metabolism in PC-3 ZIC2 -/- cells is in part due to recovered oxidative phosphorylation in the mitochondria of PC-3 ZIC2 -/- cells revealed by increased *MT-CO3* expression. Re-introduction of ZIC2 into these cells was able to significantly lower both *MT-CO3* expression and MTS assay activity. We propose that ZIC2 contributes to mitochondrial dysfunction in PC-3 cells promoting the Warburg effect and that loss of ZIC2 in PC-3 ZIC2 -/- cells contributes to an increased preference for energy production by mitochondrial oxidative phosphorylation. Data recently reported indicating that that *CD44* expression in PC-3 cells is correlated with increased glycolysis and reduced oxidative phosphorylation may provide a link between ZIC2 and mitochondrial function. CD44 mRNA levels are significantly reduced in our PC-3 ZIC2 -/- cells suggesting ZIC2 may activate *CD44* expression. The finding that ZIC2 is essential for transcriptional activation of *POU5F1* in liver cancer stem cells through interaction with the NURF

complex supports the idea that ZIC2 may act to upregulate pro-oncogenic genes such as *CD44*, *CCNA1*, *JUN* and *MMP13*.

Specifically, we verified the transcriptional upregulation of two known tumor suppressors, *KISS1* and *DKK3*, in PC-3 ZIC2 -/- cells. As its name implies, the peptide ligand *KiSS-1 metastasis-suppressor* (*KISS1*), acts as a metastasis suppressor in malignant melanomas, esophageal squamous cell, ovarian and bladder cancers through interaction with its G-protein coupled receptor.[74-75,76] Our data indicate that upregulation of *KISS1* is correlated with a loss of proliferation, migration and invasion in PC-3 ZIC2 -/- cells, which supports a metastasis-suppressor role for *KISS1* in prostate cancer. Aberrant Wnt signaling has been reported in many cancer models. Data that links ZIC2 to Wnt signaling was reported in a vertebrate model of embryonic development where ZIC2 was shown to bind to TCF4, resulting in restricted β-catenin nuclear translocation. *DKK3*, a potent Wnt signaling inhibitor, acts as a tumor suppressor by disrupting the FZD/LRP5/LRP6 receptor complex that is required for canonical WNT signaling. Our data indicating significant upregulation *DKK3* in PC-3 ZIC2 -/- cells supports the notion that *DKK3* is also tumor suppressor in prostate cancer. This is consistent with other reports of *DKK3* expression in the normal epithelium of the prostate being lost during benign and malignant transformation. Interestingly, there are conserved ZIC2 DNA binding site motifs (5'-GGTGGTC-3') near the TSS of both *DKK3* and *KISS1*.[11] Therefore, ZIC2 could conceivably function as part of a transcriptional co-repressor complex bound near the TSS of these tumor suppressor genes to downregulate their expression in lethal prostate cancer.

TMPRSS2 is found fused to ERG in ~50% of all prostate cancers. *TMPRSS2* fusions to several ETS transcription factors, including ERG, are thought to promote prostate cancer initiation when cells are androgen sensitive and AR positive. Here we report that the androgen regulated gene, *TMPRSS2*, was significantly upregulated in PC-3 ZIC2-/- cells. The parental PC-3 cells are known to be AR negative as well as androgen insensitive.[65] This suggests that some aspect of hormone regulated gene expression is compromised when ZIC2 is present and that hormone regulated gene expression is restored in PC-3 ZIC2 -/- cells as evidenced by *TMPRSS2* re-expression. The upregulation of *TMPRSS2* in PC3 ZIC2 -/- cells suggests that these cells may have re-acquired sensitivity to hormone deprivation, a trait that is ultimately lost in castration resistant prostate cancer (CRPC).

We have achieved the conclusion that ZIC2 knockdown in vitro mediates several cellular processes associated with PC-3 cells. Further studies should be done to determine the key gene targets of ZIC2 transcriptional regulation. An oncoprotein is described as a protein that influences cancer progression beyond the transcriptional level. We provide functional characteristics and molecular biology rationale for the addition of ZIC2 into the oncoprotein category. The main goal in establishing post-translational products of ZIC2 was to provide reasonable evidence for the aberrant expression of ZIC2 in prostate cancer. By introduction of K-R mutations, we first assessed transient expression of ZIC2. It was observed that K61R was not degraded in contrast to the K253R. This persuaded us to perform immunoprecipitation of ZIC2 and the predicted PTM- ubiquitination and RNF180. We have concluded that K61 is the site for ubiquitination of ZIC2 and K253 is

necessary for interaction with RNF180. Overall we have supported previous work to describe the interaction of ZIC2 and RNF180 within the first 255 amino acids of the ZIC2 sequence.

In vitro modeling in PC-3 cells was successful in proving the hypothesis that aberrant expression effects prostate cancer progression. We present Figure 23 as the working model for ZIC2 contributing to prostate cancer progression. Future studies corroborating ZIC2 will be achieved using alternative prostate cancer cell lines in vitro and in vivo modeling to establish therapeutic and clinical relevance of disrupting aberrant expression of ZIC2.

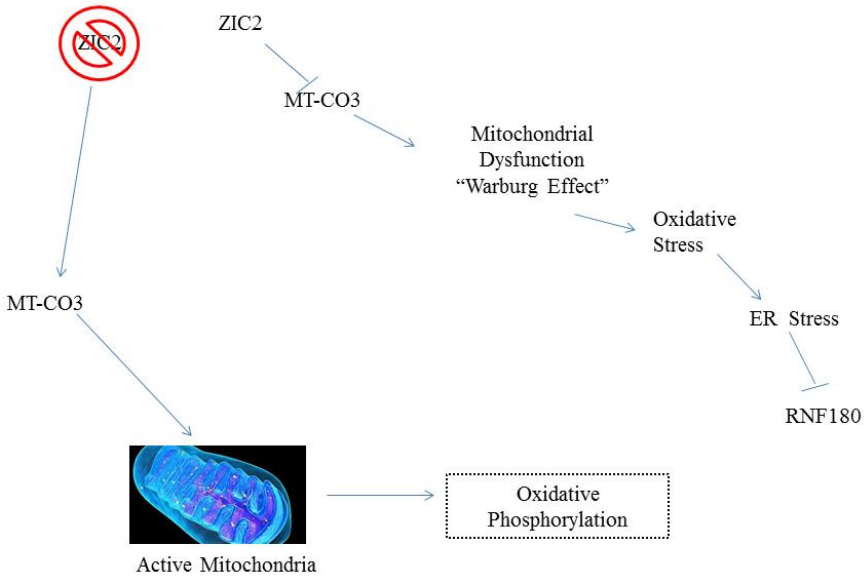


Figure 23. Working model of aberrant expression of ZIC2 contributing to prostate cancer progression.

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